

*Original Article*

**Activation of overexpressed GLP-1R attenuates prostate cancer growth by inhibiting cell cycle progression**

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## **ABSTRACT**

**Aims/Introduction:** Incretin therapy is a common treatment for type 2 diabetes mellitus.

We have previously reported an anti-prostate cancer effect of glucagon-like peptide-1 receptor (GLP-1R) agonist exendin-4. The attenuation of cell proliferation in prostate cancer cell line was dependent on GLP-1R expression. Here, we examined the relationship between human prostate cancer severity and the effect of forced expression of GLP-1R using a lentiviral vector.

**Materials and Methods:** Prostate cancer tissues were extracted by prostatectomy and biopsy. GLP-1R was overexpressed in ALVA-41 cells using a lentiviral vector (ALVA-41-GLP-1R cells). GLP-1R expression was detected by immunohistochemistry and quantitative PCR. Cell proliferation was examined by growth curves and BrdU incorporation assays. Cell cycle distribution and regulators were examined by flow cytometry and western blotting. *In vivo* experiments were performed using a xenografted model.

**Results:** GLP-1R expression levels were significantly inversely associated with the Gleason score of human prostate cancer tissues. Abundant GLP-1R expression and functions were confirmed in ALVA-41-GLP-1R cells. Exendin-4 significantly decreased ALVA-41-GLP-1R cell proliferation in a dose-dependent manner. DNA synthesis and

G1-to-S phase transition were inhibited in ALVA-41-GLP-1R cells. SKP2 expression was decreased and p27Kip1 protein was subsequently increased in ALVA-41-GLP-1R cells treated with exendin-4. *In vivo* experiments performed by implanting ALVA-41-GLP-1R cells demonstrated that exendin-4 decreased prostate cancer growth by activation of GLP-1R overexpressed in ALVA41-GLP-1R cells.

**Conclusions:** Forced expression of GLP-1R attenuates prostate cancer cell proliferation by inhibiting cell cycle progression *in vitro* and *in vivo*. Therefore, GLP-1R activation may be a potential therapy for prostate cancer.

**Key words:** GLP-1R, prostate cancer, cell cycle

## INTRODUCTION

Anti-diabetic agents mimicking incretin action, such as dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide-1 receptor (GLP-1R) agonists, have emerged as one of pivotal treatments for patients with type 2 diabetes mellitus (T2DM). Incretin action is recently taking the limelight because of their direct organ-protective effects that are provided independent on the beneficial effects associated with glucose lowering effect<sup>1</sup>. Patients with T2DM have a higher risk of cardiovascular diseases<sup>2</sup> and highly potential for restenosis after coronary angioplasty<sup>3</sup> than individuals without T2DM. Consequently, the potential of anti-diabetic treatments using incretin action to advantage not only glycaemic control but also the protection of cardiovascular system has been elucidated. Previously, we have investigated such protective effects on vascular systems including attenuation of atheroma formation in atherogenic mice<sup>4</sup> and the reduction of vascular constriction after injury<sup>5,6</sup> induced by a GLP-1R agonist exendin-4 (Ex-4). Thus, incretin therapy might be able to ameliorate quality of life and reduce mortality rates among patients with T2DM according to its vascular protection.

However, cancer, malignant neoplasms, is currently focused on as a central cause of death in patients with T2DM<sup>7</sup>. In particular, cancer has become the leading causal disease of death in Japanese patients with T2DM. The Japan Diabetes Society (JDS) and

Japan Cancer Association (JCA) have therefore issued a warning about increasing cancer risk in patients with DM<sup>8</sup>. The current Japan Diabetes Optimal Integrated Treatment study for 3 major risk factors of cardiovascular diseases (J-DOIT3) study revealed that multifactorial intensive intervention reduces cardiovascular events in Japanese patients with T2DM<sup>9</sup>. However, J-DOIT3 did not reduce the risk of all mortalities and cancer death by multifactorial intervention<sup>9</sup>. This result suggests that to establishment a new treatment strategy to reduce cancer and associated deaths for T2DM is required.

Previously, we have observed GLP-1R expression in post-operative prostate cancer tissue in non-diabetic subjects and demonstrated the attenuation of prostate cancer growth by Ex-4 via inhibiting ERK activation both *in vitro* and *in vivo*<sup>10</sup>. In addition, further reductions in tumor growth and prostate cancer cell proliferation were observed by combination treatment with Ex-4 and metformin<sup>11</sup>, without a relationship to glucose reduction. Following our experimental demonstrations, the LEADER trial, Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results, revealed that the GLP-1R agonist decreased the prostate cancer prevalence significantly in patients with T2DM, suggesting that GLP-1R agonists attenuate prostate cancer growth in not only experimental animal models, but also patients with T2DM<sup>12</sup>. In our previous report, the anti-prostate cancer effect induced by Ex-4 was dependent on GLP-1R expression in

cancer cells, and Ex-4 did not attenuate the proliferation of human prostate cancer cell line ALVA-41 that does not express endogenous GLP-1R<sup>10</sup>. To elucidate the precise anti-prostate cancer effect of GLP-1R activation, we examined the relationship between human prostate cancer severity and GLP-1R expression, and the effect of forced expression of GLP-1R using a lentiviral vector in ALVA-41 cells *in vitro* and *in vivo*.

## **MATERIALS AND METHODS**

### **Human tissues**

Human prostate cancer tissues were obtained from 30 non-diabetic subjects with prostate cancer aged from 20 to 85 years after radical prostatectomy or transrectal biopsy in Fukuoka University Hospital. The samples were embedded in paraffin, fixed in formalin, and cut into 3 µm-thick sections for immunofluorescence staining. Sections were prepared from 30 independent prostate cancers of 30 independent patients. The tissue samples were categorized into three malignancy grades by the Gleason grading system<sup>13</sup>. The Ethical Committee of Fukuoka University Hospital approved the protocol of this study (15-2-03) with opt-out consent provided by the hospital website ([http://www.hop.fukuoka-u.ac.jp/rinshou/download/PDF\(15-2-03\).pdf](http://www.hop.fukuoka-u.ac.jp/rinshou/download/PDF(15-2-03).pdf)). The present study was carried out in accordance with the principles of the Declaration of Helsinki.

## **Immunohistochemistry**

Paraffin-embedded tissue sections were stained with an anti-GLP-1R monoclonal antibody (Mab 3F52)<sup>14</sup> obtained from Novo Nordisk and an anti-P504S antibody (AN449-5ME; Biogenex, CA). Sections for GLP-1R staining were subsequently incubated with Alexa Fluor 488 goat anti-mouse IgG (A-11017, Life Technologies, CA), and sections for P504S staining were incubated with Alexa Fluor 594 goat anti-rabbit IgG (A-11012, Life technologies, CA). The sections were counterstained with DAPI and then observed by microscopy.

## **Construction of the lentiviral GLP-1R-expressing vector and transduction of cells**

We constructed a lentiviral vector including a FLAG epitope tag as described previously<sup>15</sup>, using pFLAG CMV-2 expression vector (Cat. # E7033 Sigma-Aldrich, Saint Louis, MO, USA), pLVSIN-EF1 $\alpha$  (Cat. #6186, Clontech, Mountain View, CA, USA) and 293T cells (#CRL-3216; ATCC, Manassas, VA, USA).

## **Cell culture and proliferation assay**

Cells were maintained in RPMI 1640 medium with supplementations, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cell proliferation assay was performed as described previously<sup>10</sup> with minor modifications. Briefly, cells were cultured in 12-well culture plates and maintained in medium with or without 0.1–10 nM

Ex-4 or 100 nM exendin (9–39) (**E7269**, Sigma-Aldrich), and with or without PKI<sub>14-22</sub> (**P9115**, Sigma-Aldrich). Cell proliferation ratio was counted after 0–3 days or 48 h using a hemocytometer.

## **Animals**

Male athymic CAnN.Cg-*Foxn1nu*/CrIcrlj 5 weeks aged mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and housed in a specific-pathogen-free barrier facility at Fukuoka University. The mice were subcutaneously injected with 5,000 ALVA-41-GLP-1R or ALVA-41-control cells stably expressing the *Luciferase* gene controlled the CAG promoter (catalogue no. LVP567; GenTarget Inc., San Diego, CA, USA), which were mixed with 100 µL Matrigel (Becton Dickinson, Bedford, MA, USA)<sup>15</sup>, at 6 weeks of age. Mice were treated with either saline or Ex-4 (Sigma-Aldrich, Tokyo, Japan), as we described previously<sup>10</sup>. At 10 weeks of age, tumor growth was evaluated using an IVIS Lumina In Vivo Imaging System<sup>16</sup>. After imaging, mice were euthanized and their tumors were resected. The tumor volume was calculated as we previously described<sup>10</sup>. The plasma glucose concentration was measured by Glutest Neo Super (Sanwa Chemical Co., Kanagawa, Japan). All protocols involving animals were reviewed and approved by the Animal Care Subcommittee at



Fukuoka University. All methods involving animals were performed in accordance with the relevant guidelines and regulations.

### **Reverse Transcription (RT) and quantitative real-time RT- Polymerase Chain Reaction (PCR)**

To analyze gene expression, RT and quantitative real-time PCR were performed as described previously<sup>10</sup>. Each sample was examined in triplicate and corrected by internal control, TATA-binding protein (*TBP*) mRNA expression. The primer sequences were same to our previous report<sup>10</sup>. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

### **Bromodeoxyuridine (BrdU) incorporation assay**

To evaluate the proliferation of **ALVA-control or ALVA-41-GLP-1R cells with or without Forskolin (#F6886; SIGMA-ALDRICH, Tokyo, Japan)**. BrdU assay was performed using a Cell Proliferation ELISA kit (1647229; Roche Applied Science, Mannheim, Germany), as described previously<sup>10, 11</sup>.

### **Apoptosis assay**

To detect apoptotic cells, TUNEL staining was demonstrated using the DeadEnd Fluorometric TUNEL System (Promega), according to the company's protocol, as we previously described<sup>10</sup>.

### **Measurement of the cAMP concentration**

Measurement of the cAMP concentration was performed as described previously<sup>10</sup>, using a cAMP Enzyme Immunoassay Kit (#501040; Cayman Chemical, Ann Arbor, MI, USA), according to the company's instructions.

### **Cell cycle analysis by flow cytometry**

Cell cycle analysis by flow cytometry was performed as reported previously<sup>6</sup>. Briefly, ALVA-41 cells were seeded in 60-mm dishes at  $1 \times 10^5$  cells/ml. Cells were grown to 60%–70% confluency with 10 nM Ex-4 or PBS for 48 h. Cell cycle analysis was performed using a Cycletest™ Plus DNA reagent kit (BD Biosciences Franklin Lakes, NJ, USA), following the manufacturer's instructions, and BD FACSVerse (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc., OR, USA).

### **Western blotting analysis**

Western blotting was performed as described previously<sup>6, 10</sup>. The following primary antibodies were used: phospho-ERK (Thr-202/Tyr-204) (#9101, Cell Signaling, Danvers, MA, USA), ERK (#9102, Cell Signaling), cyclin D1 (#2978, Cell Signaling), cyclin D2 (#3741, Cell Signaling), phospho-Rb (Ser807/811) (#8516, Cell Signaling),

p27Kip1 (#3686; Cell Signaling), and GAPDH (sc-20357; Santa Cruz Biotechnology, Dallas, TX, USA).

### **Statistical analysis**

The unpaired *t*-test or one-way ANOVA were performed for statistical analysis as appropriate. *P*-values of less than 0.05 were considered as statistically significant. Results are expressed as the mean  $\pm$  SEM.

## **RESULTS**

### **GLP-1R expressed in human prostate cancer is inversely associated with cancer progression**

As we reported previously<sup>10</sup>, GLP-1R is observed in prostate cancer tissue in non-diabetic subjects and colocalizes with P504S, prostate cancer marker. Interestingly, as shown in Figure 1A, Expression levels of GLP-1R were decreased in advanced prostate cancer categorized by the Gleason score<sup>13</sup>. When the 30 patients were divided into three groups according to the Gleason score, expression of GLP-1R in prostate cancer was significantly decreased in advanced prostate cancer patients with high Gleason scores compared with early stage prostate cancer patients (Figure 1B).

### **Forced expression of GLP-1R attenuates prostate cancer cell proliferation**

To elucidate the effect of GLP-1R expression on prostate cancer, we overexpressed GLP-1R in prostate cancer cells. In our previous report<sup>10</sup>, endogenous GLP-1R expression was negligibly detected in ALVA-41 cells. In the present study, we overexpressed human GLP-1R in ALVA-41 cells using a lentiviral vector. As shown in Figure 1C, *GLP1R* gene expression was abundantly detected in ALVA-41 cells transfected with the lentiviral vector carrying the human *GLP1R* gene (ALVA-41-GLP-1R cells) compared with LNCaP cells that express endogenous GLP-1R. However, GLP-1R expression was not detected in cells transfected with the empty lentiviral vector (ALVA-41-control cells). Furthermore, immunohistochemistry of GLP-1R confirmed significant membranous GLP-1R protein expression in ALVA-41-GLP-1R cells (Figure 1D). The functional effectiveness of overexpressed GLP-1R was demonstrated by intracellular cAMP induction in ALVA-41-GLP-1R cells stimulated with Ex-4 (Figure 1E). We next examined the anti-proliferative effect of GLP-1R in ALVA-41 cells. As shown in Figure 2A, the number of ALVA-41-GLP-1R cells was slightly but significantly reduced compared with ALVA-41-control cells without GLP-1R agonist treatment. In addition, Ex-4 decreased the number of ALVA-41-GLP-1R cells dose-dependently, as shown by the growth curve in Figure 2B. However, ALVA-41-control cells did not respond to Ex-4 (Figure 2C). Consistent with the growth curve data, BrdU incorporation assays

demonstrated that the proliferation of ALVA-41-GLP-1R cells was decreased significantly compared with that of ALVA-41-control cells (Figure 2D). In addition, Ex-4 attenuated ALVA-41-GLP-1R cell proliferation in a dose-dependent manner, but not ALVA-41-control cell proliferation (Figure 2E). Similar to our previous report using LNCaP cells<sup>10</sup>, GLP-1R activation did not induce apoptosis of ALVA-41-GLP-1R cells (Figure 2F).

### **Forced expression of GLP-1R attenuates cell cycle progression via inhibition of SKP2 and upregulation of p27Kip 1**

We next examined the mechanism by which overexpressed GLP-1R attenuated ALVA-41 cell proliferation. First, we conducted cell cycle analysis by flow cytometry. As shown in Figure 3A, ALVA-41-GLP-1R cells in G0/G1 phase were increased and those in S phase were decreased compared with ALVA-41-control cells. Furthermore, Ex-4 treatment decreased not only S phase entry, but also G2/M phase transition of ALVA-41-GLP-1R cells (Figure 3B). Consistent with the apoptosis assay (Figure 2F), the subG1 fraction was not observed after Ex-4 treatment, further supporting suppression of apoptosis. In Figure 3A, significantly increased G0/G1 was observed in ALVA-41-GLP-1R compared with ALVA-41-control, but not in Figure 3B. This might be an experimental limitation, because Figure 3B require 12 hours longer incubation time for PBS or Ex-4.

Because exendin (9–39), a GLP-1R antagonist (Figure 3C) and inhibitor of protein kinase A (PKA; Figure 3D), significantly counteracted Ex-4-induced attenuation of cell proliferation, Ex-4 inhibited cell proliferation via the activation of GLP-1R and cAMP-PKA signaling, a canonical pathway of GLP-1R. In our previous report using LNCaP cells, Ex-4 attenuated cell proliferation via inhibition of ERK<sup>10</sup>. However, ERK was not activated in ALVA-41 cells (Figure 3E). To confirm anti-proliferative effect of intracellular cAMP induced by GLP-1R signal, we demonstrated BrdU assay with Forskolin which is a ubiquitous activator of eukaryotic adenylyl cyclase to increase cAMP level. As shown in Figure 3F, Forskolin decreased cell proliferation in ALVA-41 cell significantly, and further reduction of cell proliferation was observed in ALVA-41-GLP-1R cell. Because G0/1 arrest was obviously induced by GLP-1R activation in ALVA-41 cells, we performed further experiments focusing on cell cycle regulators. Western blotting revealed no significant differences in Rb protein phosphorylation and cyclin D1 expression between ALVA-41-GLP-1R and ALVA-41-control cells without Ex-4 treatment (Figure 4A, B). However, p27Kip 1, a negative regulator of G0/1-to-S phase entry, was significantly increased in ALVA-41-GLP-1R cells compared with ALVA-41-control cells (Figure 4C). Furthermore, Ex-4 treatment significantly decreased Rb phosphorylation (Figure 4D) and cyclin D1 expression (Figure 4E), and significantly

increased p27Kip1 expression (Figure 4F) in ALVA-41-GLP-1R cells, but not in ALVA-41-control cells. Because p21Kip1 protein levels are post-translationally regulated by SKP2 ubiquitin ligase, we next examined SKP2 expression by quantitative RT-PCR. As shown in Figure 4G, *SKP2* gene expression was decreased significantly by Ex-4 in ALVA-41-GLP-1R cells, but not in ALVA-41-control cells.

### **Forced expression of GLP-1R attenuates prostate cancer growth *in vivo* independent of glucose metabolism**

To determine the anti-prostate cancer effect of overexpressed GLP-1R *in vivo*, we implanted ALVA-41-GLP-1R and ALVA-41-control cells, which stably expressed cytomegalovirus-luciferase (CAG-Luc), as reported previously<sup>16</sup>, into athymic nude mice. Four weeks after subcutaneously implantation of ALVA-41 cells into the flank region of mice, tumor formation was visualized by *in vivo* imaging of the fluorescence intensity derived from CAG-Luc in ALVA-41 cells just prior to sacrifice (Figure 5A). The tumor growth measured by the fluorescence intensity of ALVA-41-GLP-1R cells was decreased compared with that of ALVA-41-control cells without Ex-4, but it was not statistically significant. However, Ex-4 treatment significantly reduced the tumor growth of ALVA-41-GLP-1R cells compared with that of ALVA-41-control cells without Ex-4 (Figure 5B). In resected tumors, the calculated tumor growth of ALVA-41-GLP-1R cells was

attenuated, but it was not statistically significant (Figure 5C). However, the tumor weight of ALVA-41-GLP-1R cells was decreased significantly compared with that of ALVA-41-control cells without Ex-4, and Ex-4 treatment significantly decreased the tumor weight of ALVA-41-GLP-1R cells comparing with that of ALVA-41-control cells treated with Ex-4 (Figure 5D). During the experimental period, serum glucose levels and body weights were not significantly different between the four groups (Figure 5E, F).

## DISCUSSION

We investigated that expression of GLP-1R in human prostate cancer cells was inversely associated with cancer progression, and that forced expression of GLP-1R inhibited prostate cancer cell proliferation *in vivo* and *in vitro* by attenuating cell cycle progression, in the present study. Incretin therapies have recently emerged as major anti-diabetic agents in the world<sup>17</sup> including Japan<sup>18</sup>. Several advantages of incretin therapy, such as a protection of pancreatic  $\beta$ -cells, possibility of body weight loss, and fewer hypoglycemic events, have been reported<sup>19</sup>. Furthermore, incretin therapy is one of the therapeutic options for T2DM even in chronic renal failure<sup>20</sup>. In addition, recent large-scale randomized control trials have suggested that GLP-1R agonists significantly reduced cardiovascular events<sup>21,22</sup>. Following these evidences, the early use of GLP-1R



agonists is recommended by the American Diabetes Association, especially for patients with established atherosclerotic cardiovascular and chronic kidney diseases<sup>23</sup>. However, the currently emerging consideration for incretin therapies should be their long-term guaranteed safety, including the cancer risk.

The mechanism-of-action of GLP-1 on cancer is still under elucidation, as we described in a previous review<sup>24</sup>. Although some data have indicated a risk of carcinogenesis by GLP-1R agonist use, there is no evidence that GLP-1R agonists increase cancer onset or death in randomized control trials. Nonetheless, we have previously investigated anti-cancer effects of a GLP-1R agonist in not only prostate cancer models<sup>10, 11</sup>, but also in breast cancer<sup>15</sup> models. These data suggest anti-cancer effects of GLP-1R agonists. Among numerous cancers associated with DM and metabolic syndromes<sup>25</sup>, the association between prostate cancer and DM is controversial, and some data suggest that patients with T2DM have a lower risk of prostate cancer compared with non-diabetic subjects<sup>26</sup>. However, a higher incidence of prostate cancer has been observed in large-scale studies conducted in Western countries<sup>12</sup> and in Japan also<sup>9</sup>. Further, a higher BMI and higher plasma C-peptide concentrations increase prostate cancer mortality<sup>27</sup>. Previously, we have demonstrated that insulin-like growth factor-I (IGF-I) and insulin increase prostate cancer cell proliferation *in vitro*<sup>28</sup>. These data suggest that

caution is needed regarding prostate cancer, especially in obese and insulin-resistant patients with T2DM. Expression level of GLP-1R was inversely associated with the Gleason score and prostate cancer advances (Figure 1A, B), in human prostate cancer. These data indicate that the activation of GLP-1R could be a marker of early stage prostate cancer, and that GLP-1R agonist may be a therapeutic option for patients with T2DM complicated with early stage prostate cancer based on our previous report<sup>10</sup> and the present study. In our earlier study, Ex-4 attenuated LNCaP cell proliferation by inhibiting ERK activation<sup>10</sup>. However, in ALVA-41 cells, ERK was not activated (Figure 3E). As clearly explained in review article<sup>29</sup>, ERK is one of the most important growth signal in prostate cancer. However, ALVA-41 does not have ERK, probably because ALVA-41 is a cell line from bony metastasis from human prostate cancer, not primary prostate cancer<sup>30</sup>, and some transformation might happen in growth signal. In addition, cAMP response element binding protein (CREB) is one of the most major transcriptional factor activated by ERK phosphorylation, and we have previously demonstrated that Ex-4 decreased CREB phosphorylation in vascular smooth muscle cell<sup>6</sup>. While CREB was slightly detected in ALVA-41 cell, phosphorylation of CREB was not detected and modified in ALVA-41 cell with or without GLP-1R expression and Ex-4 treatment (Supplementary Figure 1). Further, to confirm anti-prostate cancer effect of GLP-1R, we

demonstrated forced expression of GLP-1R in PC3 cell, a popular prostate cancer cell line. As shown in Supplementary Figure 2, Ex-4 attenuated cell proliferation in PC3 cell force-expressed GLP-1R. Interestingly, overexpression of GLP-1R and treatment with Ex-4 attenuated ALVA-41 cell proliferation by inhibiting cell cycle progression, which was independent of ERK activation, suggesting that GLP-1R activation attenuates cell proliferation by activating different signaling pathways depending on the cell types. In the present study, GLP-1R activation decreased SKP2 expression and subsequently increased p27Kip 1 protein levels to induce G0/1 arrest and inhibit cell cycle progression. In fact, we have reported a similar effect of Ex-4 in vascular smooth muscle cells (VSMCs)<sup>6</sup>. Ex-4 inhibited SKP2 expression and attenuated VSMC proliferation and neointima formation after vascular injury<sup>6</sup>. These data suggest that SKP2 might be a critical regulator of the anti-proliferative effect of GLP-1R activation in proliferating cells such as cancer cells and VSMCs. SKP2 is an F box protein that regulates p27Kip 1 ubiquitination and degradation, functioning as an ubiquitin ligase<sup>31</sup>. Interestingly, an opposing interaction between the GLP-1 action and SKP2 has been reported by another study<sup>32</sup>. GLP-1 upregulates SKP2 expression and additionally downregulates p27Kip 1 expression to accelerate cell proliferation through an insulin receptor substrate 2-dependent signal transduction in pancreatic  $\beta$ -cells<sup>32</sup>. The interaction between GLP-1

signaling and SKP2 is most likely influenced by cell proliferative activity and other growth signals. In the present study, key mechanism by which Ex-4 attenuated ALVA-41 cell via GLP-1R activation could be upregulation of cAMP level, because Forskolin also decreased ALVA-41 cell proliferation. In fact, it is reported that cAMP activates SKP2 expression and attenuates cell proliferation in vascular smooth muscle cell<sup>33</sup>. SKP2 induction by cAMP increased by GLP-1R signal could be one of mechanism by which GLP-1 attenuates prostate cancer growth, similar to ERK inhibition<sup>10</sup>. In addition, an association between GLP-1R and prostate cancer has not been investigated by genome-wide research. Although relationships between variants of *TCF7L2* (*transcription factor 7-like 2*), one the most important transcription factors for *GLP-1R* expression, and some cancers, such as breast, colorectal and lung cancers, have been reported<sup>34</sup>, further elucidation is required.

Considering primary action of GLP-1, GLP-1R agonists are anti-diabetic agent which induce anti-apoptotic action and cell proliferation of pancreatic  $\beta$ -cells via ERK, Akt and  $\beta$ -catenin activation<sup>35</sup>. However, the present data and our previous reports<sup>10, 11, 15</sup> suggested anti-proliferative effect of GLP-1R agonist in cancer cells. These two actions seem opposite effect. Among cancer cells, Koehler et al. reported that GLP-1R agonist Ex-4 does not modify cell growth and apoptosis in pancreatic cancer cells<sup>36</sup>. GLP-1 action

and downstream signal of GLP-1R might be different depending on cell line and cancerous or not cancerous cell. Further elucidation about GLP-1R signal depending on cell background and cross talking with other signal transductions should be required.

In conclusion, we investigated an anti-prostate cancer effect by overexpressed GLP-1R activation *in vitro* and *in vivo*. The present study may facilitate establishing diabetes therapies to prevent cancer, and GLP-1R activation may be an option for prostate cancer therapy.

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## **DISCLOSURE**

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## FIGURE LEGENDS

### **Figure 1. GLP-1R expression in human prostate cancer and overexpression of human GLP-1R in ALVA-41 cells.**

A. Immunohistochemistry of GLP-1R and P504S was performed to examine GLP-1R expression in human prostate cancer tissues obtained by prostate gland resection or biopsy. Staining is representative of prostate cancer tissues from 10 independent non-diabetic patients. Sections were stained with anti-GLP-1R or P504S antibodies and counterstained with DAPI (magnification,  $\times 400$ ). B. GLP-1R-positive cells and cancerous P504S-positive cells were counted in four individual fields of view. One-way ANOVA was performed to calculate statistical significance (\*\* $P < 0.01$  vs. Gleason 6,  $^{\#}P < 0.05$  vs. Gleason 7). C. RT-PCR of the coding sequence of human *GLPIR* was performed to detect

*GLP1R* expression. *TBP* was used as the internal control. D. Immunohistochemistry was performed to detect expression of human GLP-1R in ALVA-41 and LNCaP cells. E. The intracellular cAMP concentration was measured in ALVA-41-control and ALVA-41-GLP-1R cells with or without Ex-4 stimulation. The unpaired *t*-test was performed to calculate statistical significance (\*\* $P < 0.01$  vs. 0 min) (n=3).

**Figure 2. Attenuation of prostate cancer cell proliferation by overexpression of GLP-1R and Ex-4 stimulation.**

Growth curves of ALVA-41-control and ALVA41-GLP-1R cells without Ex-4 A, ALVA-41-GLP-1R cells with or without Ex-4 B, and ALVA-41-control cells with or without Ex-4 C. A. The unpaired *t*-test was performed to calculate statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$  vs. ALVA-control) (n=3). B, C. One-way ANOVA was performed to calculate statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$  vs. PBS) (n=3). D, E. BrdU assays were performed to measure DNA synthesis in ALVA-41-control and ALVA-41-GLP-1R cells with or without Ex-4 for 24 h. Data are expressed as relative absorbance to ALVA-41-control D and 0 nM Ex-4 in ALVA41-control or ALVA-41-GLP-1R cells. The unpaired *t*-test was performed to calculate statistical significance. D. \* $P < 0.05$  vs. ALVA-41-control (n=3). E. \* $P < 0.05$ , \*\* $P < 0.01$  vs. 0 nM Ex-4 (n=3).

**Figure 3. Cell cycle distribution and signal transduction.**

A, B Flow cytometric analysis was performed to determine the cell cycle distribution of ALVA-41-control and ALVA-41-GLP-1R cells with or without Ex-4. Data are represented as the ratios of cells distributed in each phase to the total cells. The unpaired *t*-test was performed to calculate statistical significance. A. \**P*<0.05 vs. ALVA-41-control (n=3). B \**P*<0.05 vs. ALVA-41-control and #*P*<0.05, ##*P*<0.01 vs. ALVA-41-GLP-1R+PBS (n=3). C, D. Growth curves of ALVA-41-GLP-1R cells with or without Ex-4, Ex9–39, or PKI. The unpaired *t*-test was performed to calculate statistical significance for C \**P*<0.05 vs. PBS and #*P*<0.05 vs. Ex-4+Ex9–39 (n=3), and D \**P*<0.05 vs. PBS and #*P*<0.05 vs. Ex-4+PKI (n=3). E. Western blotting of phospho-ERK, ERK, and GAPDH was performed in three independent ALVA-41 cell lysate samples. F. BrdU assay were performed to measure DNA synthesis in ALVA-41-control and ALVA-41-GLP-1R with or without Forskolin (FK) for 24 h. Data are expressed as relative absorbance compared with 0μM Forskolin in ALVA41-control or ALVA-41-GLP-1R. One-way ANOVA with a post hoc Dunnett's test was performed to calculate statistical significance. \**P*<0.05, \*\**P*<0.01 vs. EtOH (0μM) (n=5).

**Figure 4. Expression of cell cycle regulators in ALVA-41-GLP-1R cells.**

Western blotting of A, D phosphorylated Rb, B, E cyclin D1, and C, F p27Kip 1 was performed in ALVA-41-control and ALVA-41-GLP-1R cells with or without 10 nM Ex-4 for 24 h. Densitometry was performed by normalization to GAPDH. Data are represented as relative expression to ALVA-41-control cells A–C or cells treated with PBS D–G. Quantitative real-time RT-PCR of *SKP2* was performed in ALVA-41-control and ALVA-41-GLP-1R cells with or without 10 nM Ex-4 for 24 h. The unpaired *t*-test was performed to calculate statistical significance. C. \**P*<0.05 vs. ALVA-41-control D–G. \**P*<0.05 vs. ALVA-41-GLP-1R treated with PBS.

**Figure 5. Forced expression of GLP-1R attenuates prostate cancer growth *in vivo*.**

A. ALVA-41-control or ALVA-41-GLP-1R cells stably transfected with the *Luciferase* gene were implanted into athymic nude mice with or without Ex-4 treatment. Tumor growth was visualized using an *in vivo* imaging system. B. Quantification of fluorescence were determined in tumor cells. C. Tumor volumes were calculated by the modified ellipsoid formula. D. Tumor weight was measured by balance. E. Plasma glucose and F. body weight were measured during the experimental period. The unpaired *t*-test was performed to calculate statistical significance: \**P* < 0.05 vs. ALVA-41-control+PBS, #*P*<0.05 vs. ALVA-41-control+Ex-4.



Figure 1

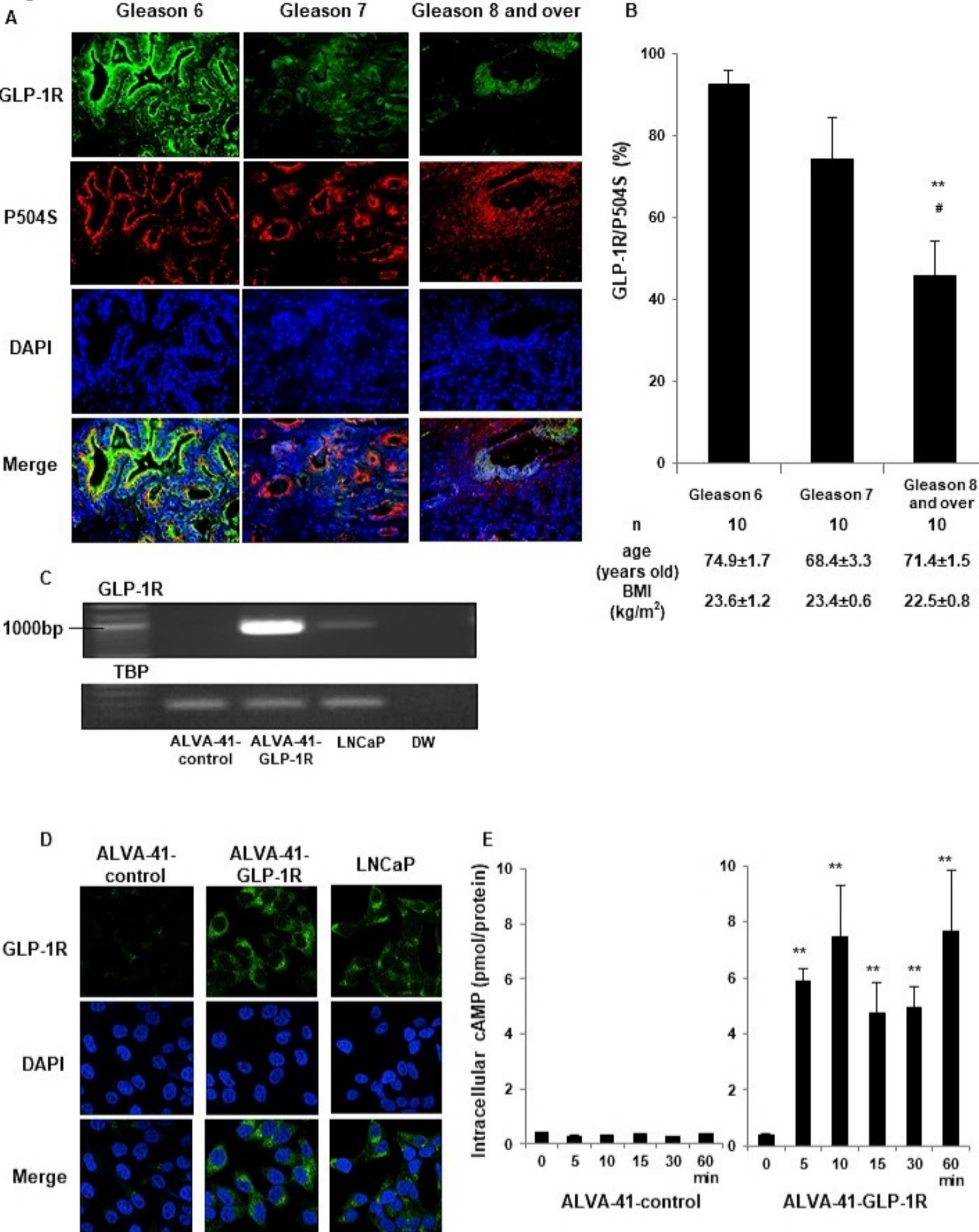


Figure 2

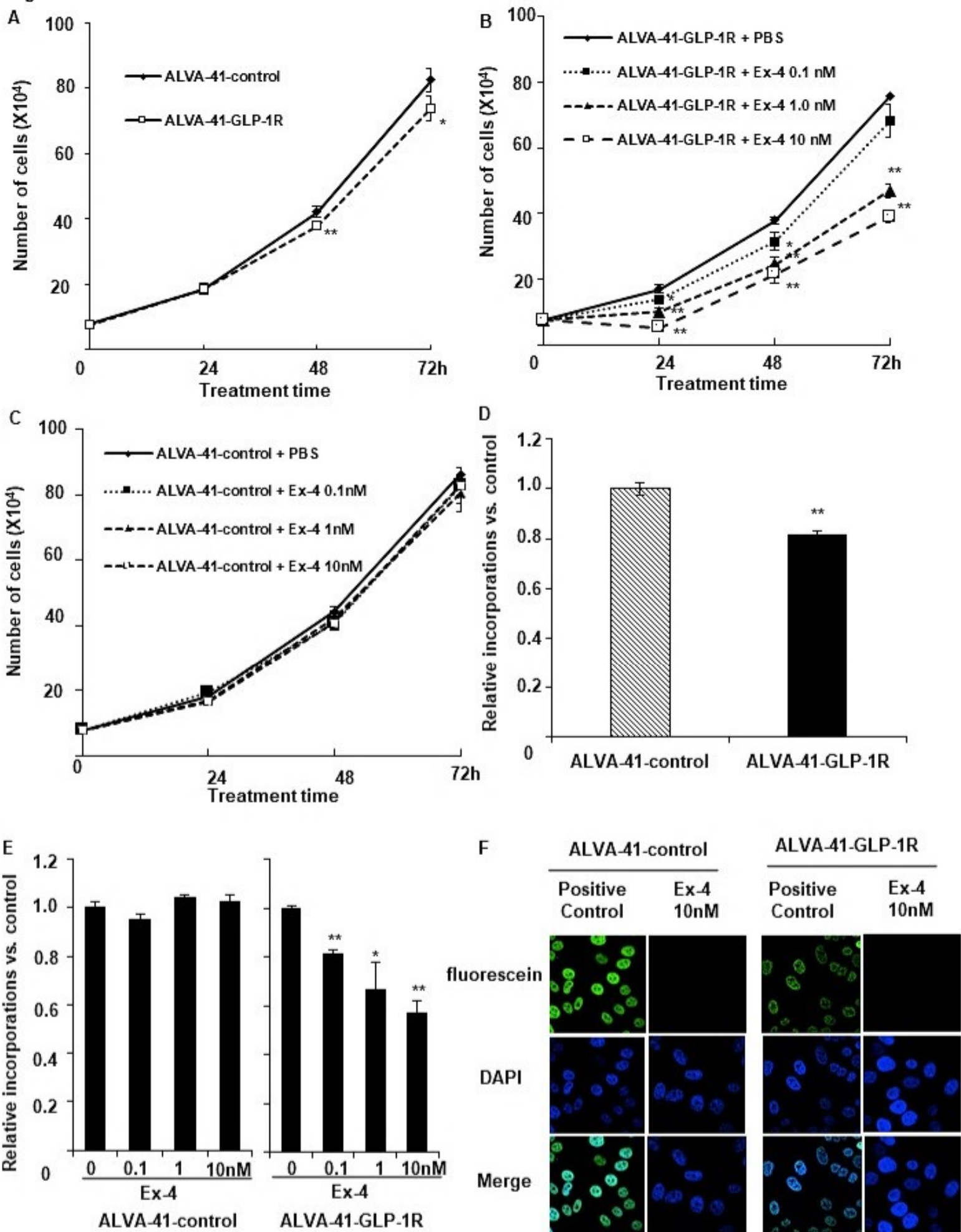
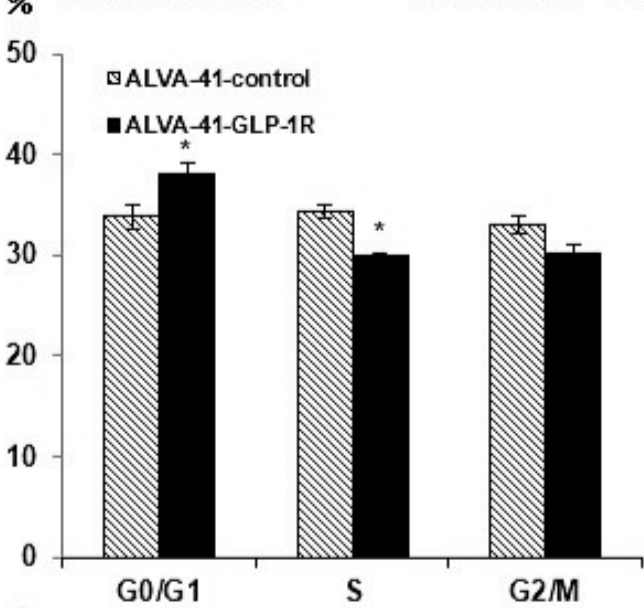
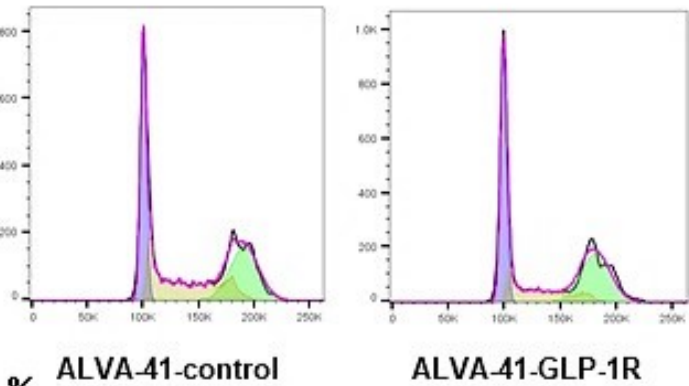


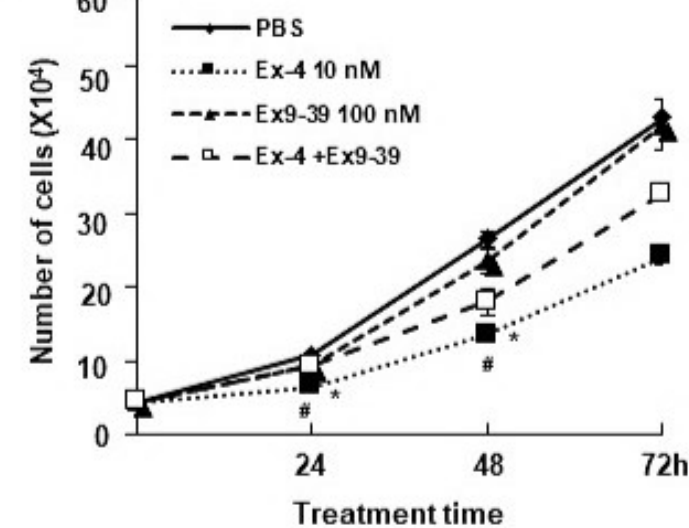


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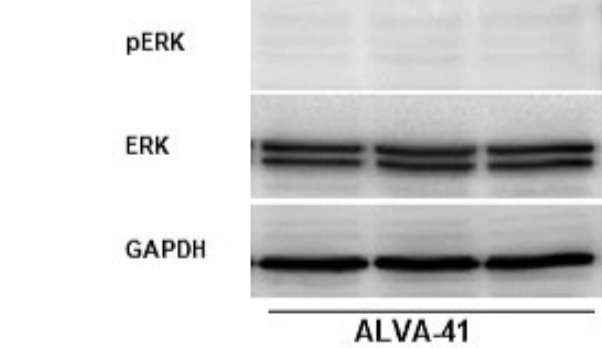
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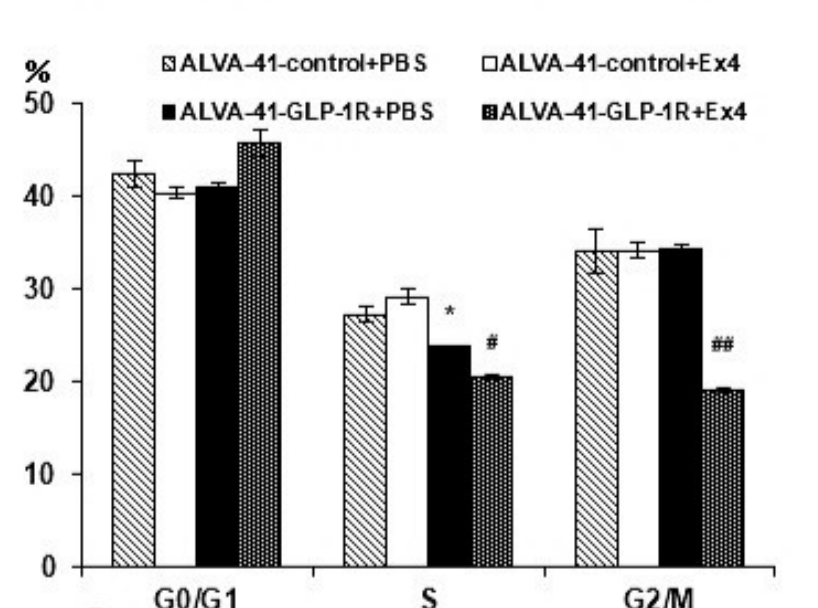
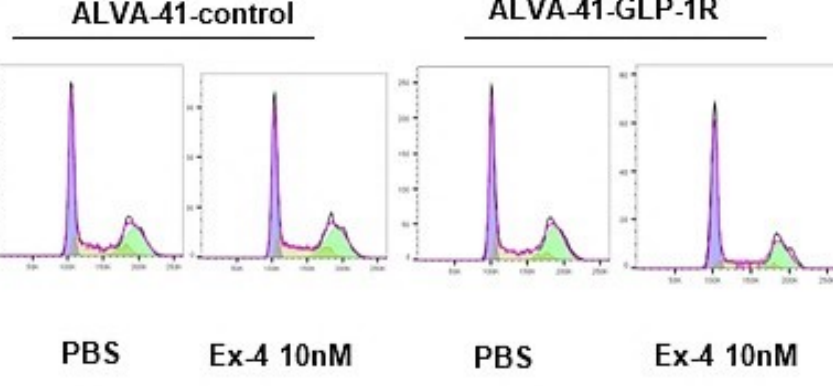
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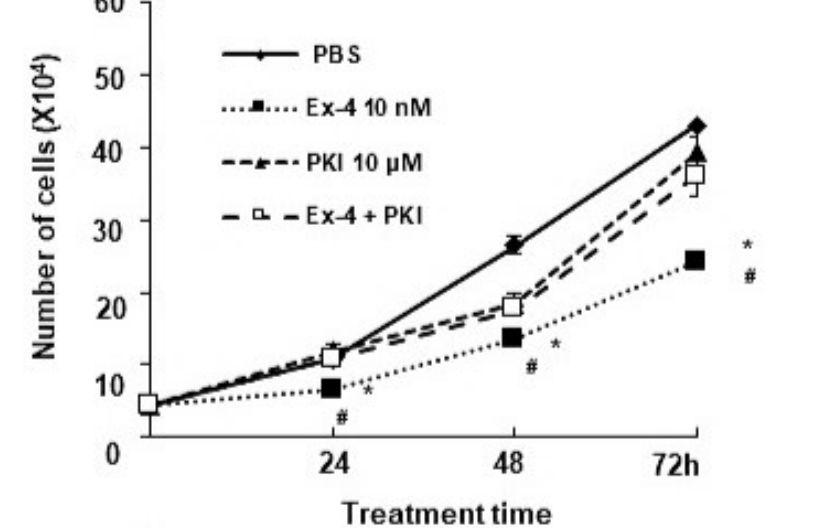
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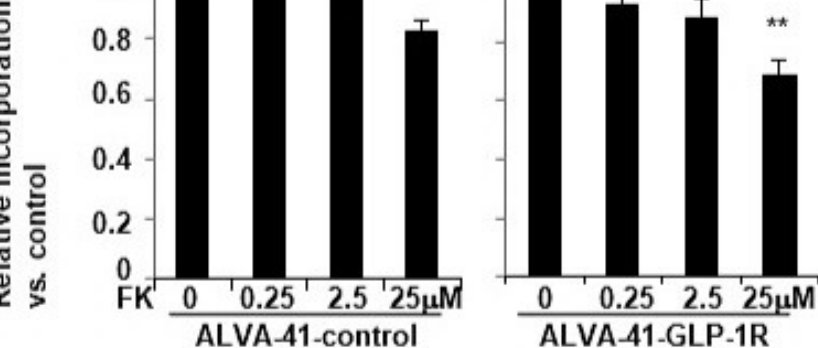




Figure 4

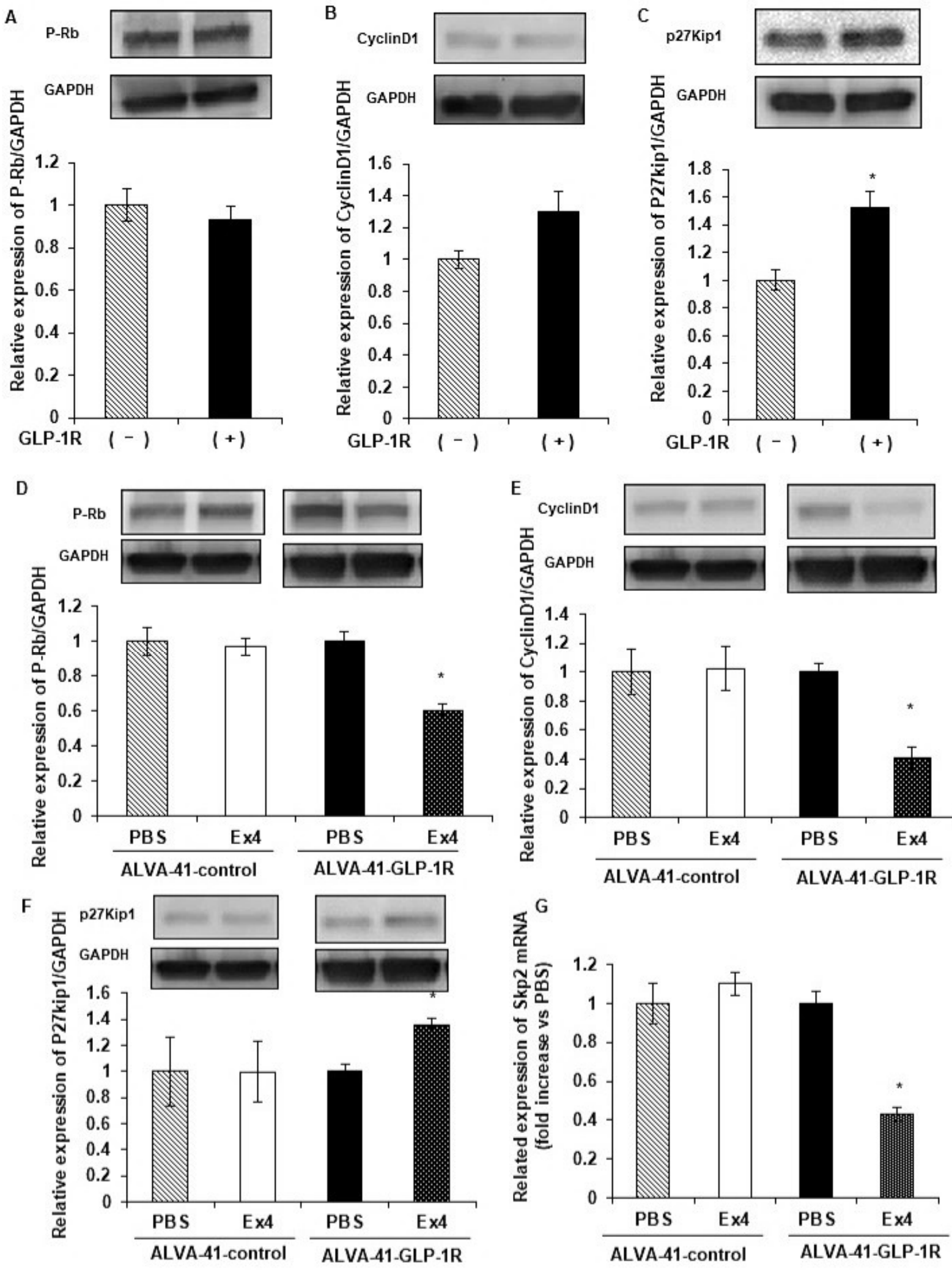
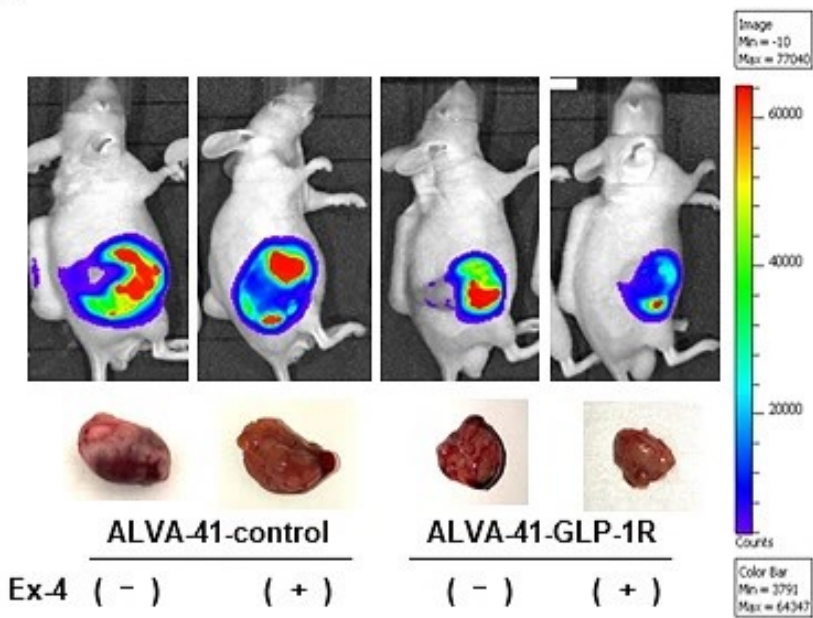
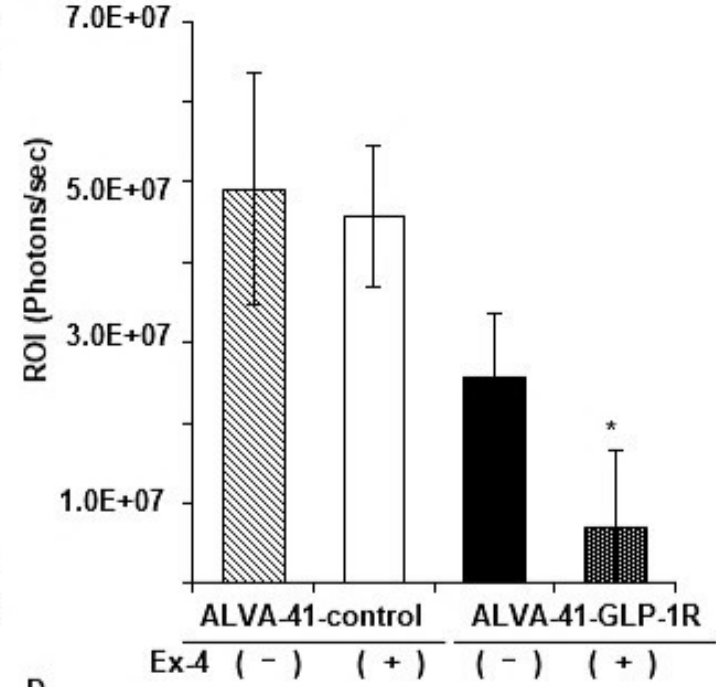


Figure 5

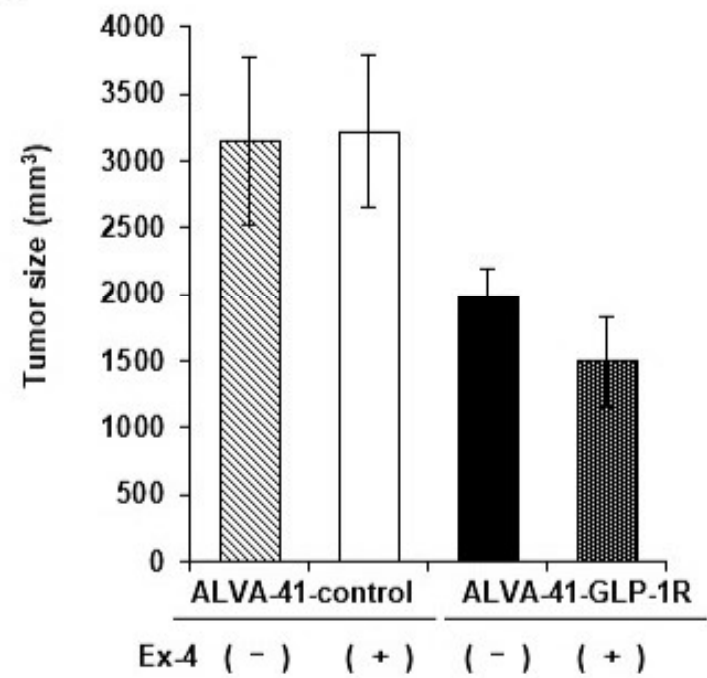
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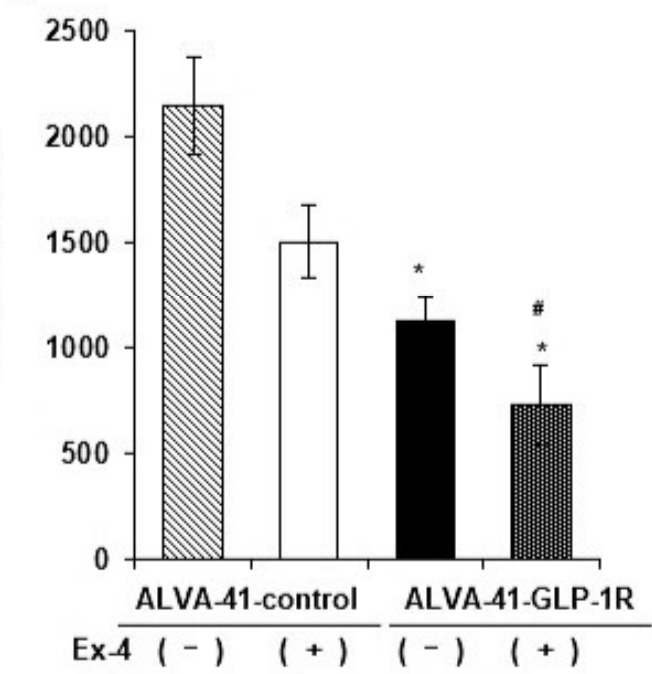
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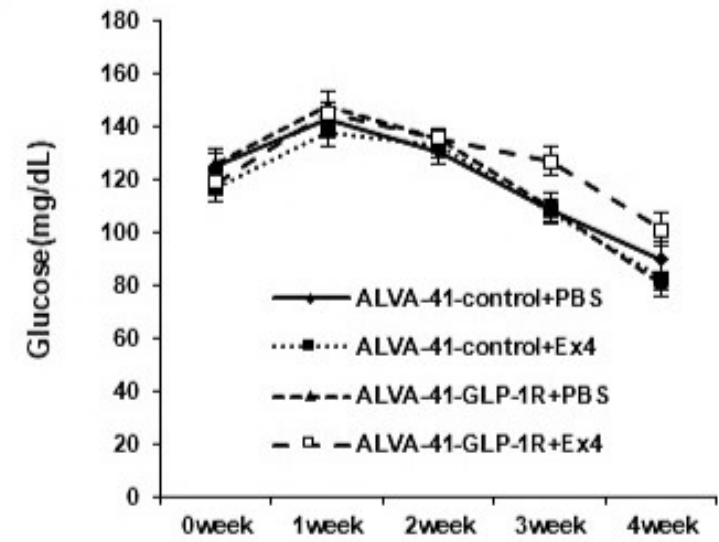
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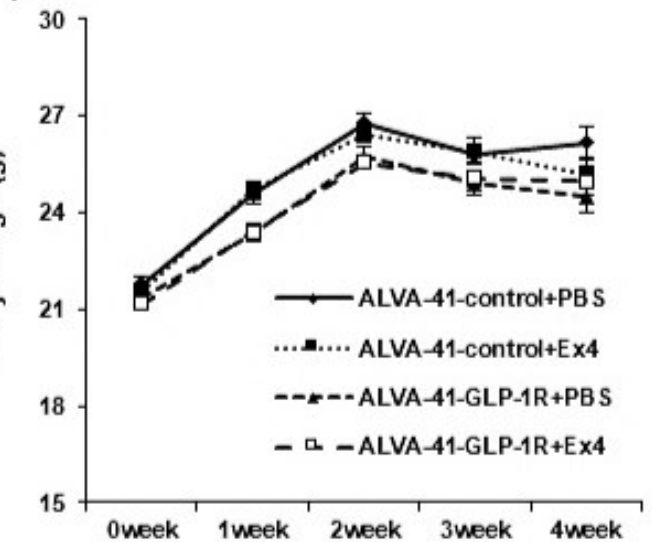
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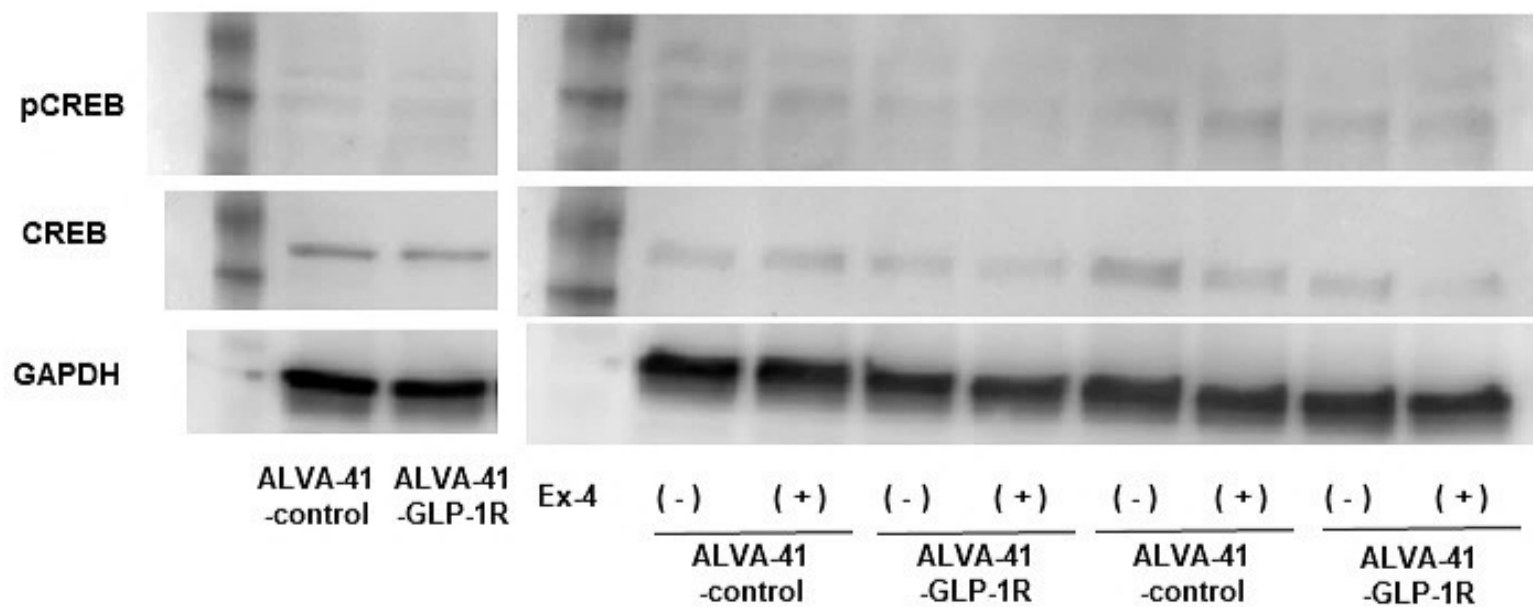


F



**Supplementary Figure 1. CREB expression and phosphorylation in ALVA-41 cells.**

Western blotting of phospho-CREB(pCREB), CREB, and GAPDH was performed in ALVA-41-control and ALVA-41-GLP-1R with or without 10nM Ex-4 for 24 hr. The following primary antibodies were used: phospho-CREB (Ser133) (#9198, Cell Signaling, Danvers, MA, USA), CREB (#9197, Cell Signaling) and GAPDH (sc-20357; Santa Cruz Biotechnology, Dallas, TX, USA).



**Supplementary Figure 2. Overexpression of human GLP-1R in PC3 cells.**

PC3 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM Nutrient Mixture F-12. Human GLP-1R was overexpressed as described in Methods. (a) Immunohistochemistry was performed to detect expression of human GLP-1R in PC3 cells. (b) BrdU assay were performed to measure DNA synthesis in PC3-control and PC3-GLP-1R with or without Ex-4 for 24 h. Data are expressed as relative absorbance compared with 0nM Ex-4. Unpaired *t*-tests were performed to calculate statistical significance. \**P*<0.05, \*\**P*<0.01 vs. 0nM Ex-4 (n=3).

